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## **Introduction**

Breast cancer initiation and progression involves the serial accumulation of a variety of genetic (DNA) alterations to the cell (1). One such event involves loss of heterozygosity (LOH) of DNA which has been shown to occur frequently in primary breast cancers and more so in metastatic disease (2). It has been suggested that these allelic losses associated with malignancy may involve potential tumor suppressor genes and/or metastasis regulator genes (3,4). Detection of these circulating tumor-associated DNA markers in the blood from cancer patients may serve as surrogate markers of subclinical disease progression and permit identification of high-risk patients early in their disease course (4-6). Furthermore DNA based genetic testing of serial blood samples offers an easily accessible route by which to evaluate ongoing genetic events which may have prognostic significance (7). This new approach provides for a minimally invasive method to monitor subclinical disease progression and offers a novel technique for potentially assessing response to therapeutic interventions in early stage breast cancer patients (8). We have successfully demonstrated the detection of circulating DNA microsatellites for LOH in the serum of breast cancer patients (9). We will be developing a methodology to allow for high throughput and multiplexing of DNA markers in order to more efficiently and accurately evaluate multiple samples and establish a molecular marker profile for each individual patient. Assays for LOH have been converted to capillary array electrophoresis (CAE).

Our studies were put on hold by the Office of Regulatory Compliance and Quality, U.S. Army Medical Research and Materiel Command for a year due to "semantics" of the protocol and patient consent form which were approved by our local Institutional Review Board over two years ago. We had revised and resubmitted the protocol and patient consent form in January of 2003 but were told that they will not be reviewed until Ms. Dillner is able to clear up her other obligations. This hold continued on until November 2003. It is not until recently that the protocol and consent form were finally reviewed and our studies allowed to be continued. Due to the interruption, our research was brought to a standstill. We will now resume from where we left off in June 2002.

## **BODY**

### **Aim Ia: Assessment of microsatellite markers for LOH in serum.**

In this aim, we will assess breast cancer patients' serum for LOH using a panel of eight polymorphic microsatellite markers (TP53, D16S421, D17S855, D17S849, D14S5, D14S62, D10S197 and D8S321) on five chromosomes known to demonstrate frequent LOH in primary breast tumors. DNA will be isolated from serum obtained pre-operatively from AJCC stage I-III patients and at the time of diagnosis from AJCC stage IV patients. In addition, paired lymphocyte DNA will be isolated from each patient to serve as a normal control. Following PCR, fragments were separated by gel electrophoresis and LOH assessed using a fluorescent scanner and ClaritySC 3.0 software (Media Cybergenetics,

Silver Spring, MD). We have now converted the post-PCR fragment separation and detection to a capillary array electrophoresis (CAE) (Beckman CEQ 8000 XL) assay. We have compared the assay sensitivity and specificity between the gel electrophoresis/fluorescent scanner and the CAE. The sensitivity and throughput capacity are much improved using the CAE. Samples previously assessed by gel electrophoresis were validated by the CAE. Samples are to be run in triplicate. Serum will be collected from 30 healthy female donors and assessed in a similar manner.

**Aim Ib. Assessment of LOH markers in fluid of bone marrow aspirations.**

For this aim, because bone is such a common site of breast cancer recurrence, we sought to determine whether microsatellite markers associated with breast cancer could be detected in BM aspirates from patients with early stage breast cancer. Cell-free plasma from BM aspirates in 48 patients was collected intra-operatively from patients undergoing surgery for their primary breast cancer diagnosis. This is a retrospective study of BM aspirates previously collected. Eight polymorphic microsatellite markers which correspond to regions that have been shown to demonstrate significant LOH suggesting sites of putative tumor suppressor and/or metastasis related genes were selected: D1S228 at 1p36; D8S321 at 8qter-8q24.13; D10S197 at 10p12; D14S51 at 14q32.1-14q32.2; D14S62 at 14q32; D16S421 at 16q22.1; D17S849 at 17pter-17qter and D17S855 at 17q. Methods are being developed to optimize extraction of DNA from BM plasma. DNA was isolated from acellular fluid obtained from BM aspirations, purified and quantified. Paired lymphocytes were collected from each patient and DNA extracted to serve as normal control for each PCR reaction. Following PCR, fragments will be assessed using the CAE assay system.

**Aim Ic. Correlation of LOH microsatellite markers in paired bone marrow and primary tumors.**

To determine whether a correlation existed between LOH detected in early stage breast cancer patients' bone marrow and primary tumors, we plan to assess match-paired specimens with identical microsatellite markers. DNA will be isolated from paired primary tumor and bone marrow specimens and analyzed using 8 microsatellite markers (D1S228, D8S321, D10S197, D14S51, D14S62, D16S421, D17S849 and D17S855). These samples will be assessed by CAE.

**Aim IIa. Assessment of circulating tumor cells in blood by qualitative RT-PCR and correlation to LOH in serum**

In this study, we will assess blood from breast cancer patients diagnosed with breast cancer. RNA was extracted from cells in the blood and will be analyzed using a quantitative Realtime RT-PCR assay. The previously developed assay using ultrasensitive electrochemiluminescence detection solution phase technology (IGEN) with specific probes for the presence of four tumor markers ( $\beta$ -HCG, c-MET, GalNAc and MAGE-3) will be validated by the quantitative Realtime RT-PCR assay. Furthermore, we are currently in the process of collecting and assessing matched-paired serum samples for LOH and

circulating tumor cells in the blood by qualitative and quantitative RT-PCR for correlation.

**Aim IIb. Assessment of circulating tumor cells in blood by quantitative RT-PCR**

Currently we are developing and optimizing markers used in IIA for quantitative RealTime RT-PCR (qRT). Primers, probes and standards have been developed. The assays for individual markers are being evaluated on controls for specificity and sensitivity. The optimized assays will be used for assessment of blood from patients.

**Aim IIc. Assessment of isolated tumor cells in blood**

We will be investigating apoptotic cells in culture and tissue sections by immunostaining. The objective is to develop an optimized assay that can be informative and reliable. We find that single cells are difficult to assess for apoptosis whereby, clumps of cells are easier to assess. Currently we are developing techniques to improve isolation of tumor cells from blood. Different types of magnetic beads and antibodies are being tested to get optimal cell isolation.

**Aim IIIa. Neoadjuvant treatment analysis**

To date we have not analyzed patients receiving neoadjuvant treatment.

**KEY RESEARCH ACCOMPLISHMENTS**

- Conversion of the gel electrophoresis/fluorescent scanner assay for assessment of microsatellite to capillary array electrophoresis (CAE).
- Conversion of IGEN electrochemiluminescence RT-PCR detection assay to a quantitative Realtime RT-PCR assay.

**REPORTABLE OUTCOMES**

**A. Manuscripts**

1. Taback B, Giuliano AE, Hansen NM, Singer FR, Shu S, Hoon D. Detection of Tumor-Specific Genetic Alterations in Bone Marrow from Early Stage Breast Cancer Patients. Cancer Research, 63: 1884-1997, 2003.

**B. Abstracts**

None.

## CONCLUSIONS

Since June 2002, we have been put on hold by the Office of Regulatory Compliance and Quality, U.S. Army Medical Research and Materiel Command, therefore there has been very limited research or collection of specimens for this study.

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## APPENDICES

1. Taback B, Giuliano AE, Hansen NM, Singer FR, Shu S, Hoon D. Detection of Tumor-Specific Genetic Alterations in Bone Marrow from Early Stage Breast Cancer Patients. *Cancer Research*, 63: 1884-1887, 2003.

# Detection of Tumor-Specific Genetic Alterations in Bone Marrow from Early-Stage Breast Cancer Patients<sup>1</sup>

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## ABSTRACT

Detection of genetic markers associated with early breast cancer may prove clinically relevant for identifying patients at increased risk for relapse. Loss of heterozygosity (LOH), a specific genetic aberration, commonly occurs during breast cancer initiation and metastasis. Early detection of tumor metastasis to bone marrow (BM) using conventional histochemical techniques has been limited because of suboptimal efficiency and sensitivity. Because bone is such a common site of breast cancer recurrence, we sought to determine whether microsatellite markers associated with breast cancer could be detected in BM aspirates from patients with early-stage breast cancer. Cell-free plasma from BM aspirates in 48 patients was assessed for LOH using a panel of eight polymorphic microsatellite markers. LOH was detected in 11 (23%) of 48 patients' BM aspirates. Advancing American Joint Committee on Cancer (AJCC) stage was associated with an increased incidence of LOH. Concordance was present between LOH identified in BM aspirates and matched-pair primary tumors. No samples contained detectable tumor cells on routine histology. In 24 patients, paired peripheral blood serum samples were available for analysis. In these cases, BM detection of LOH was more frequent than serum. This study demonstrates the novel finding of tumor-related genetic markers in BM aspirate plasma of early-stage breast cancer patients and provides a unique approach for assessing subclinical systemic disease progression and the monitoring of breast cancer patients.

## INTRODUCTION

Bone is the most frequent site of systemic spread of breast cancer (1, 2). Once metastases are clinically apparent, overall prognosis is poor. Undetected occult tumor cells contribute to disease recurrence, and therefore, methods to identify subclinical disease (micrometastasis) may improve staging and guide additional therapeutic decisions. Historically, conventional cytological assessment of blood and BM<sup>3</sup> aspirates has been performed with limited success (3, 4). Immunocytochemical techniques using antibodies specific to epithelial antigens have improved sensitivity and can identify a single tumor cell among a background of >1 million normal cells (5, 6). Enrichment methods with antibody-magnetic bead conjugates of BM aspirates have demonstrated the presence of occult tumor cells in early-stage breast cancer patients (5, 7). Recently, it has been shown that detection of micrometastasis in the BM of early-stage breast cancer patients is an independent prognostic risk factor (8, 9). However, accurate microscopic analysis of many cytological samples requires considerable cytopathologic expertise and can be tedious, particularly if performed serially to assess disease progression and or response to treatment. Additionally, the variable specificity of individual antibodies

used to detect single cells has been questioned (8-11). Finally, these assay systems cannot characterize the biological behavior of the cells being detected and thus many may represent dormant tumor cells, apoptotic cells, nonpathologic tumor cells, or displaced normal breast epithelial cells.

A variety of serial genetic changes have been implicated in the initiation and progression of solid tumors. One such event, allelic imbalance (LOH), has been shown to occur commonly in primary breast tumors and with additional frequency in metastasis (12-15). Furthermore, there is emerging evidence to suggest that microsatellite markers for detecting LOH at specific chromosome loci may have important clinical prognostic correlations (12, 16, 17). However, examination of an excised primary tumor specimen may be of limited value in that it provides information of those genetic events that have occurred and not ongoing alterations that may be of clinical relevance, either prognostically or for therapeutic decisions. Additionally, because of the potentially long latent period that may exist between early breast cancer diagnosis and clinically detectable systemic recurrence, improved assessment methods are needed for serial surveillance of occult disease progression and monitoring response to therapy.

Recently, it has been shown that free tumor-associated DNA can be identified in the serum and plasma from patients with melanoma, breast, lung, renal, gastrointestinal, and head and neck tumors (18-32). Furthermore, a high-quality concordance has been shown to exist between the genetic alterations (*i.e.*, LOH, microsatellite instability, mutations) found in circulating tumor DNA and those from the primary tumor suggesting a potential surrogate tumor marker (20-22, 27, 28). Early studies have shown prognostic importance of circulating microsatellite markers for LOH in blood (21, 24). Because BM is a common site for breast cancer recurrence, we sought to determine whether BM aspirate plasma could provide a viable source to detect tumor-specific DNA associated with systemic metastasis from early-stage breast cancers.

## MATERIALS AND METHODS

**Surgical Specimens and DNA Isolation.** BM aspirates were collected in 4.5-ml sodium citrate tubes (Becton Dickinson, Franklin Lakes, NJ) through bilateral anterior iliac approach from 48 consecutive patients as follows: ductal carcinoma *in situ*, 1 patient; AJCC stage I, 32 patients; AJCC stage II, 13 patients; and AJCC stage III, 2 patients undergoing surgical resection of their primary breast cancer at the Saint John's Health Center/John Wayne Cancer Institute. In addition, 5 healthy female volunteer donors contributed BM aspirate samples for controls. All patients signed an informed consent form approved by the Saint John's Health Center/John Wayne Cancer Institute Institutional Review Board. BM was drawn, and (cell-free supernatant) plasma was immediately separated by centrifugation (1000 × *g*, 15 min), filtered through a 13-mm serum filter (Fisher Scientific, Pittsburgh, PA) to remove any potential contaminating cells, aliquoted, and cryopreserved at -30°C. In addition, in 24 patients matched-paired, peripheral venous blood (6 ml) was drawn preoperatively, and serum was harvested in the aforementioned manner. For normal genomic DNA controls, whole blood was collected from each patient spotted and stored on FTA blood cards (Fitzco, Minneapolis, MN) before DNA isolation. DNA was extracted from 1 ml of BM aspirate plasma and peripheral blood serum using QIAamp extraction kit (Qiagen, Valencia, CA) as described previously (23).

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<sup>3</sup>The abbreviations used are: BM, bone marrow; LOH, loss of heterozygosity; AJCC, American Joint Committee on Cancer.



To assess the correlation of LOH found in the BM and that of the primary breast tumor, DNA was isolated from 10- $\mu$ m sections cut from paraffin-embedded tissue blocks. Samples were deparaffinized, microdissected using laser capture microscopy (Arcturus, Mountain View, CA) from normal tissue, and incubated with proteinase K in lysis buffer [50 mM Tris-HCl, 1 mM EDTA, and 0.5% Tween 20] at 37°C overnight and then heated at 95°C for 10 min.

Additionally, each BM aspirate was assessed for the presence of occult tumor cells by conventional histological staining methods using H&E.

**Microsatellite Markers and PCR Amplification.** Eight polymorphic microsatellite markers that correspond to regions that have been shown to demonstrate significant LOH, suggesting sites of putative tumor suppressor and/or metastasis related genes were selected: D1S228 at 1p36; D8S321 at 8qter-8q24.13; D10S197 at 10p12; D14S51 at 14q32.1-14q32.2; D14S62 at 14q32; D16S421 at 16q22.1; D17S849 at 17pter-17qter; and D17S855 at 17q. All primer sets were obtained from Research Genetics (Huntsville, AL), and sense primers were labeled with a fluorescent dye: 5-(and 6-)carboxyfluorescein. Approximately 20 ng of genomic DNA was amplified by PCR in 25- $\mu$ l reactions containing 1 $\times$  PCR buffer (Perkin-Elmer, Foster City, CA), 6 pmol of each primer, 1 unit of Taq DNA polymerase, 2.5  $\mu$ M deoxynucleotide triphosphates, and 1.5 mM MgCl<sub>2</sub>. Forty PCR cycles were performed with each cycle consisting of 30 s at 94°C, 30 s at 50°C–56°C, and 90 s at 72°C, followed by a final extension step of 72°C for 5 min as described previously (23).

**LOH Analysis.** PCR products were electrophoresed on 6% denaturing polyacrylamide gel containing 7.7 M urea at 1600 V for 2 h. Genomix SC scanner (Beckman Coulter, Fullerton, CA) was used to image the fluorescent-labeled PCR products, and densitometric analysis was performed with ClaritySC software (Media Cybernetics, Silver Spring, MD). Intensity calculations and comparisons of the specific alleles in patients' normal control and respective BM DNA were performed to evaluate for LOH. The LOH was defined if a >50% reduction of intensity was noted in one allele from tumor or BM DNA when compared with the respective allele in the matched-paired lymphocytes (23).

Clinical and histopathological data were obtained from patient chart review and the Breast Tumor Registry at the John Wayne Cancer Institute.  $\chi^2$  and Wilcoxon rank-sum tests were performed for statistical evaluation of association of BM LOH status and known prognostic parameters.

## RESULTS

DNA from BM aspirate plasma samples were isolated and purified. Eight microsatellite markers representing six chromosomes known to have frequent LOH in primary breast tumors were assessed. LOH was identified in 11 (23%) of 48 patients' BM aspirates. LOH was most commonly identified at microsatellite marker D14S62 occurring in 4 (12%) of 34 informative patients. Microsatellite markers demonstrating LOH at D1S228 and D14S51 occurred in 3 (8%) of 38 informative patients each, followed by LOH at D8S321 (5%), D10S197 (4%), and D17S855 (3%). No LOH was detected for microsatellite markers D16S421 and D17S849 (Table 1). Eight of 11 patients with detectable LOH in their BM demonstrated this event at only one of the chromosome loci assessed, and 3 patients (1 stage I, 2 stage II patients) contained LOH for two microsatellite markers. No LOH was detected for any of the microsatellite markers assessed in the patient with ductal carcinoma *in situ* or the BM aspirates collected from 5 healthy female donors.

There was an increased association between the presence of LOH in

Table 2 Association of LOH in patients' BM aspirates with AJCC stage

AJCC stage	Patients with LOH in BM/total patients (%)
I	6/32 (19%)
II	4/13 (31%)
III	1/2 (50%)

the BM and advanced disease stage. Six (19%) of 32 AJCC stage I patients demonstrated LOH for at least one marker, in contrast to 4 (31%) of 13 AJCC stage II patients, and 1 (50%) of 2 AJCC stage III patients (Table 2). Ten clinicopathological prognostic factors were assessed for correlation with BM LOH status: histological tumor type; size; grade; Bloom-Richardson score; lymph node involvement; presence of lymphovascular invasion in the primary tumor; receptor status (estrogen, progesterone, HER2); and p53 status. There was an association between larger tumor size and BM LOH positivity: 2.46 *versus* 1.81 cm, mean tumor sizes, respectively. There was also a trend toward an increased incidence of BM LOH in lymph node-positive patients as compared with lymph node negative patients: 3 (33%) of 9 patients *versus* 8 (21%) of 38 patients, respectively. However, no correlation reached statistical significance between any of the clinicopathological prognostic factors and BM LOH status in this pilot study, except histology. Lobular carcinomas were more likely associated with increased LOH in BM aspirates than infiltrating ductal tumors: 6 (60%) of 10 patients *versus* 5 (14%) of 37 patients, respectively ( $\chi^2$  test,  $P = 0.006$ ). Larger populations with long-term follow-up are warranted to evaluate the clinical and prognostic utility of this assay.

To determine whether a correlation existed between the LOH detected in patients BM and their primary tumor, DNA was isolated from primary tumors and evaluated with identical microsatellite markers. Ten of 11 patients demonstrating LOH in their BM had primary tumors available for assessment. In all 10 patients, the LOH identified in the BM was also present respectively in the primary tumor (Fig. 1).

Conventional histological analysis of all specimens using standard H&E staining did not demonstrate occult tumor cells in any of the BM samples. Finally, in 24 patients, paired serum from peripheral venous blood was available for assessment. In this group, LOH was identified in the BM aspirates of 5 (21%) patients. One patient demonstrated the same LOH pattern in the paired serum sample, and in another patient, LOH was detected in only one of two markers positive in the BM. A third patient demonstrated LOH in their serum sample for one marker but no LOH in their BM. In all other cases, no LOH was detected in peripherally collected serum samples.

## DISCUSSION

There is mounting evidence to suggest that the presence of occult tumor cells in the BM of breast cancer patients may have prognostic significance (9, 33–38). Furthermore, some have shown these findings to be independent of pathologic lymph node status (8, 9). These studies are important because historically 20% of lymph node-negative patients will subsequently develop systemic disease, and therefore, early detection of BM micrometastasis may identify high-risk patients for additional systemic therapy. More so, BM provides a readily accessible source to serially monitor subclinical disease progression and the potential impact of adjuvant therapies early in the disease course. Conventional histological analysis of BM aspirates for tumor cells has proven unreliable (3, 4). More recently, immunocytochemical techniques using antibodies to epithelial antigens expressed on tumor cells have improved detection sensitivity. However, assay reliability has been shown to be highly dependent on the antibody selected, as well as the variability by which the tumor cell

Table 1 LOH frequency in breast cancer patients' BM aspirates

Microsatellite marker	LOH in BM aspirates/informative cases (%)
D14S62	4/34 (12%)
D14S51	3/38 (8%)
D1S228	3/38 (8%)
D8S321	2/39 (5%)
D10S197	1/26 (4%)
D17S855	1/37 (3%)
D17S849	0/31 (0%)
D16S421	0/28 (0%)

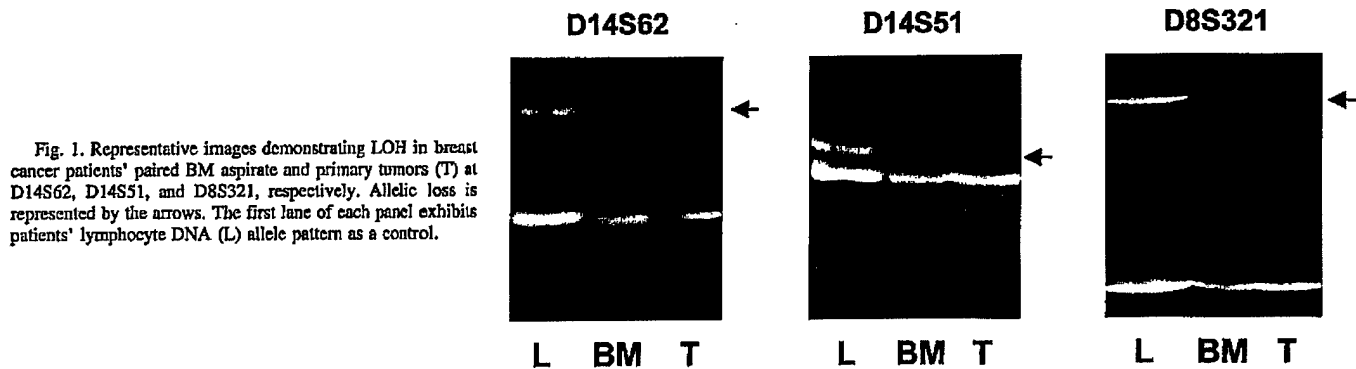


Fig. 1. Representative images demonstrating LOH in breast cancer patients' paired BM aspirate and primary tumors (T) at D14S62, D14S51, and D8S321, respectively. Allelic loss is represented by the arrows. The first lane of each panel exhibits patients' lymphocyte DNA (L) allele pattern as a control.

expresses the preferred epitope (8, 11). Finally, sample processing and antibody staining require considerable attention to methodology and an experienced reviewer to interpret the results.

With the implication of an accrual of aberrant genetic events in tumor development and progression and their potential for clonality, these genetic markers may provide unique surrogates for monitoring subclinical disease events, particularly in light of the ease and widespread use of PCR techniques. Studies have demonstrated the presence of circulating nucleic acids in the plasma and serum of patients with various malignancies (32). In breast cancer, LOH presence in plasma/serum has been described to occur anywhere from 15–66% (19, 21–23, 30). These results may vary because of differences in techniques of sample collection and processing, DNA isolation, PCR methods, and scoring of LOH. Furthermore, we have shown that the presence of circulating tumor DNA increases with advancing stage of disease (23). More so, because blood is rapidly circulating and an inhospitable environment and BM is a frequent site of breast cancer relapse, we sought to determine whether BM aspirates may harbor tumor-specific DNA alterations associated with early breast cancer progression.

In this study, we have developed a highly sensitive method of detecting tumor-specific DNA in the BM aspirates of breast cancer patients. The increased incidence in more advanced stages correlates with tumor burden and, therefore, may have applicability as a surrogate marker for disease detection, prognosis and monitoring tumor progression, and response to therapy. Although there was an association between known prognostic factors in breast cancer (tumor size, lymph node status, and AJCC stage) and BM LOH in this initial report, additional studies are under way to address these findings in a larger cohort of patients for clinically statistical significance. The advantage of this approach is its ease of use as an assessment tool and its broad application to a variety of malignancies. Identification of additional tumor-specific genetic markers or combinations thereof may additionally enhance the assay's use. Stable tumor-specific DNA markers that accurately reflect minimal disease states, correlate with tumor progression, and that can be readily measured are highly desired. The genetic-based diagnostic test we have described is highly sensitive and specific. This approach may provide a unique alternative/supplement to optical systems for occult tumor detection that can be technically demanding and viewer dependent or those methods (reverse transcription-PCR) that assess mRNA markers that may have limited specificity because of unstable gene products, variable expression levels, and nonspecific transcripts (39–42). Although the source of this free DNA is unknown (*i.e.*, tumor cells in BM, blood, or the primary tumor), detection of genomic alterations in BM can be significant and may prove more specific than immunohistochemical and/or current mRNA marker assays. Larger studies are presently underway to evaluate these findings and determine clinicopathological correlations.

We found LOH on chromosome 14q as the most frequent event identified on circulating DNA in BM. In a previous study, LOH on 14q has been shown to occur more commonly in primary tumors without lymph node metastasis, suggesting a site for a possible metastasis related gene, however, the metastasis itself was not assessed for LOH (43). One explanation is that metastatic clones at different sites may demonstrate different LOH profiles. Additionally, differences in these results may reflect the stability of this marker as detected from various sources (blood, BM, tumor tissues), or it may be uniquely associated with site-specific metastasis. Molecular markers that are specific for the metastatic phenotype and/or sites of metastasis may prove useful for focusing clinical assessments.

Our incidence of LOH ranged from 0 to 12% for any of the microsatellite markers for informative cases. Similar detection of LOH has been described from the peripheral plasma/serum of early-stage breast cancer patients (19, 22, 23). For 10 of 11 patients whose BM contained LOH, primary tumor blocks were available for assessment, and in all cases, a similar corresponding LOH pattern was identified in the respective primary tumor specimens. The findings demonstrate the specificity of this marker detection system. Moreover, no patients had detectable tumor cells identified on routine histopathologic examination. This demonstrates the relative ease and sensitivity DNA detection assays pose as potential surrogates of subclinical disease. More importantly, BM LOH was more prominent than that of serum LOH, suggesting that assessing this site may have clinical use. BM is a frequent site of breast cancer metastases and may serve as a homing site or static reservoir for more accurate evaluations than peripheral blood, which is transient. Because of earlier detection of breast cancers and the benefits of adjuvant chemotherapy in these stages, the implications of these findings will require long-term follow-up for clinical correlation. However, it must be emphasized that a need exists for improved methods of occult disease surveillance to more accurately assess individual patient risk and modify treatment strategies accordingly before clinical manifestations occur.

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